Impairment in Ischemia-Induced Neovascularization in Diabetes

Bone Marrow Mononuclear Cell Dysfunction and Therapeutic Potential of Placenta Growth Factor Treatment

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Mechanisms that hinder ischemia-induced neovascularization in diabetes remain poorly understood. We hypothesized that endogenous bone marrow mononuclear cell (BM-MNC) dysfunction may contribute to the abrogated postischemic revascularization reaction associated with diabetes. We first analyzed the effect of diabetes (streptozotocin, 40 mg/kg) on BM-MNC pro-angiogenic potential in a model of surgically induced hindlimb ischemia. In nondiabetic animals, transplantation of BM-MNCs isolated from nondiabetic animals raised the ischemic/nonischemic angiographic score, capillary number, and blood flow recovery by 1.8-, 2.7-, and 2.2-fold, respectively, over that of PBS-injected nondiabetic animals ($P < 0.05$). Administration of diabetic BM-MNCs also improved the neovascularization reaction in ischemic hindlimbs of nondiabetic mice but to a lesser extent from that observed with nondiabetic BM-MNC transplantation. In diabetic mice, injection of nondiabetic BM-MNCs was still more efficient than that of diabetic BM-MNCs. Such BM-MNC dysfunction was associated with the impairment of diabetic BM-MNC capacity to differentiate into endothelial progenitor cells (EPCs) *in vitro* and to participate in vascular-like structure formation in a subcutaneous Matrigel plug. Placenta growth factor (PlGF) administration improved by six-fold the number of EPCs differentiated from diabetic BM-MNCs *in vitro* and enhanced ischemic/nonischemic angiographic score, capillary number and blood flow recovery by 1.9-, 1.5- and 1.6-fold, respectively, over that of untreated diabetic animals ($P < 0.01$). Endogenous BM-MNC pro-angiogenic potential was affected in diabetes. Therapeutic strategy based on

PlGF administration restored such defects and improved postischemic neovascularization in diabetic mice. (*Am J Pathol* 2004, 164:457–466)

The ability of organisms to spontaneously develop collateral vessels represents an important response to vascular occlusive diseases that determines in part the severity of residual tissue ischemia. Initially, ischemia-induced neovascularization was thought to solely result of the angiogenic process which involved hypoxia and inflammation-related pathways.^{1–4} However, recent studies demonstrate that postnatal neovascularization does not rely exclusively on sprouting of pre-existing vessels, but also involves bone marrow-derived progenitor cells. In the setting of ischemia, a subset of endothelial cell precursor (including human CD34-expressing cells and mouse sca-1-expressing cells) was shown to promote vessel growth.^{5,6} Moreover, endothelial progenitor cells (EPCs) can be grown out of isolated CD34-positive cells *in vitro* and make a significant contribution to blood vessel formation.^{7,8} Finally, the rise in BM-derived progenitor cells levels may contribute to the pro-angiogenic effect of growth factors, such as vascular endothelial growth factor (VEGF) or granulocyte macrophage-colony stimulating factor.^{5,9} Recently, placenta growth factor (PlGF) and its receptor VEGF-R1 (Flt-1) have also been shown to enhance the mobilization of myeloid progenitors into the peripheral blood and their infiltration into inflamed tissues, highlighting the potential for PlGF administration to modulate bone marrow derived cells function.¹⁰ Treatment with bone marrow-derived cells may provide a useful novel therapeutic strategy to improve postnatal angiogenesis. In this view, administration of BM-derived mononuclear cells (BM-MNCs) secrete potent angio-

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genic ligands and cytokines and supply EPCs which enhance collateral perfusion in ischemic tissue of diabetic and nondiabetic animals.^{11–13} Similarly, autologous implantation of BM-MNCs in patients with peripheral arterial disease increases the angiogenic response and improves pain-free walking time.¹⁴ Finally, injection of CD133⁺ bone marrow cells into the infarct border zone improves infarct tissue perfusion in patients with acute transmural myocardial infarction.¹⁵

Diabetics commonly suffer cardiovascular complications, including vascular diseases. In diabetic patients, collateralization is insufficient to overcome the loss of blood flow through occluded arteries leading to ischemia and often nontraumatic limb amputation. However, only few studies focus on the identification of factors that may affect neovascularization in the setting of ischemia in diabetes. It has been suggested that alteration in VEGF expression and signalization or inflammation-related pathway may reduce blood vessels formation in diabetes.^{16,17} Alternatively, EPC proliferation and adhesion ability were affected in patients with type II diabetes suggesting that BM-derived cells may have altered functions in the setting of diabetes.¹⁸ We therefore hypothesized that the impairment of ischemia-induced neovascularization observed in diabetes may be related to a decrease in the pro-angiogenic potential of endogenous BM-MNCs. In the present proposal, we 1) evaluated the pro-angiogenic potential of diabetic BM-MNCs by assessing the effect of administration of BM-MNCs isolated from nondiabetic and diabetic mice on revascularization in the ischemic hindlimb of nondiabetic mice; 2) examined the putative influence of diabetic environment by evaluating the effect of administration of BM-MNCs isolated from nondiabetic and diabetic mice on revascularization in the ischemic hindlimb of diabetic mice; and 3) attempted to analyze the mechanisms involved in diabetes-induced impairment in BM-MNC pro-angiogenic activity. To this aim, we examined the effect of diabetes on BM-MNC differentiation into EPCs *in vitro* and on the ability of BM-MNCs to participate in vascular-like structure formation; 4) assessed the therapeutic potential of PIGF administration on diabetes-induced BM-MNC dysfunction and impairment of postischemic neovascularization.

Materials and Methods

Experimental Protocol

Induction of Diabetes

To induce moderate diabetes, C57Bl/6 mice (8 weeks old, Iffa Creddo, Lyon, France) were injected intraperitoneally with 40 mg/kg of streptozotocin (STZ; Sigma Chemical Co) in 0.05 mol/L Na citrate, pH 4.5, daily for 5 days. Three days after the fifth injection, blood glucose levels were measured. If serum glucose was less than 9 mmol/L, mice were injected for an additional twice a week at the same dosage. Glucose levels were retested again every week to ensure serum glucose level of greater than

10 mmol/L in mice. Mice with glucose levels less than 10 mmol/L were excluded from further study. In the nondiabetic groups, mice were injected intraperitoneally with 0.05 mol/L Na citrate, pH 4.5.

Administration of BM-MNCs

After 2 months, right femoral artery ligation was performed as previously described.^{3,4} Using this surgical procedure and using C57Bl6 mice, we did not observe autoamputation in nondiabetic or diabetic animals. At the time of ischemia, nondiabetic and diabetic mice received intravenous injections of 1×10^6 BM-MNCs or PBS. BM-MNCs were also labeled with a green fluorescent marker, PKH2-GL.¹³ Animals were sacrificed at day 10 after ischemia.

Therapeutic angiogenesis

In a second set of experiments, an osmotic minipump (subcutaneously implanted on the backs of the mice; Alzet, type 2001, Cupertino, CA) delivered for 14 day a daily dose of 1.5 μ g of PIGF (R&D Systems, Minneapolis, MN) in nondiabetic and diabetic mice. At day 7, ischemia was induced by right femoral artery ligation and animals were sacrificed at day 14.

Quantification of Angiogenesis

Microangiography

Vessel density was evaluated by high definition microangiography as previously described.^{3,4} Briefly, mice were anesthetized (isoflurane inhalation) and a contrast medium (barium sulfate, 1 g/ml) was injected through a catheter introduced into the abdominal aorta. Images acquired by a digital X-ray transducer were assembled to obtain a complete view of the hindlimbs. The vessel density was expressed as a percentage of pixels per image occupied by vessels in the quantification area. A quantification zone was delineated by the place of the ligation on the femoral artery, the knee, the edge of the femur and the external limit of the leg.

Capillary Density

Microangiographic analysis was completed by assessment of capillary densities in ischemic and nonischemic muscles at day 10 as previously described.^{3,4} Frozen tissue sections from gastrocnemius muscle (7 μ m) were incubated with rabbit polyclonal antibody directed against total fibronectin (dilution 1:50, TEBU, Yvelines, France) to identify capillaries. Capillaries were revealed with a fluorescent FITC anti-rabbit antibody (dilution 1:10). Capillary density were analyzed in three different sections of gastrocnemius muscle. Five different fields were used in each section (Histolab software, Microvision Instrument, Evry, France). Capillary/myocyte ratios were calculated and the results were then expressed according to ischemic/nonischemic ratios.

Figure 1. A: Representative microangiography of the right ischemic and left nonischemic hindlimbs in nondiabetic mice treated with BM-MNCs isolated from nondiabetic or diabetic mice. **B:** Ischemic/nonischemic angiographic score at 10 days following ischemic injury in diabetic and nondiabetic mice treated with BM-MNCs. Values are means $\pm$ SEM, $n = 7$ per group. * $P < 0.05$ versus PBS-injected nondiabetic animals. $^{\dagger}P < 0.05$ versus mice treated with nondiabetic BM-MNCs, $^{\#}P < 0.05$ versus PBS-injected diabetic animals. PBS indicates mice receiving PBS; nondiabetic BM-MNCs, animals treated with BM-MNCs isolated from nondiabetic animals and diabetic BM-MNCs, animals treated with BM-MNCs isolated from diabetic mice.

Laser Doppler Perfusion Imaging

To provide functional evidence for ischemia-induced changes in vascularization, Laser Doppler perfusion imaging experiments were performed at day 10 as previously described.^{3,4} Briefly, excess hairs were removed by depilatory cream from the limb, and mice were placed on a heating plate at 37°C to minimize temperature variation. Nevertheless, to account for variables, including ambient light, temperature, and experimental procedures, blood flow was calculated in the foot and expressed as a ratio of ischemic to nonischemic leg. After right femoral artery ligation, mice showing less than 80% of blood flow reduction were excluded from this study.

Isolation of Mouse BM-MNCs

Bone marrow cells were obtained by flushing the tibias and femurs of nondiabetic and diabetic male C57Bl/6

		Ischemic	Non-Ischemic	Ischemic/Non-ischemic
Non-diabetic mice	PBS	1.61 $\pm$ 0.22	5.11 $\pm$ 0.60	0.31 $\pm$ 0.04
	Non-diabetic BM-MNCs	4.32 $\pm$ 0.51**	5.32 $\pm$ 0.71	0.81 $\pm$ 0.10**
	Diabetic BM-MNCs	2.64 $\pm$ 0.41* †	5.23 $\pm$ 0.61	0.54 $\pm$ 0.07* †
diabetic mice	PBS	0.92 $\pm$ 0.074	4.81 $\pm$ 0.71	0.19 $\pm$ 0.06
	Non-diabetic BM-MNCs	2.94 $\pm$ 0.40##	4.92 $\pm$ 0.83	0.60 $\pm$ 0.09##
	Diabetic BM-MNCs	1.94 $\pm$ 0.32# †	4.71 $\pm$ 0.82	0.42 $\pm$ 0.06# †

Capillary density expressed as a ratio of capillary number/fibre number. * $p < 0.05$, ** $p < 0.01$ versus PBS non diabetic mice; $^{\dagger}p < 0.05$ versus non diabetic BM-MNCs; # $p < 0.05$, ## $p < 0.01$ versus PBS diabetic mice

Figure 2. A: Representative photomicrographs of ischemic muscle sections from nondiabetic mice treated with BM-MNCs isolated from nondiabetic or diabetic mice, hybridized with antibody directed against total fibronectin. Capillaries appear in white and myocytes in black. **B:** Ischemic and nonischemic capillary density. Values are means $\pm$ SEM, $n = 7$ per group. * $P < 0.05$, ** $P < 0.01$ versus PBS-injected nondiabetic animals; $^{\dagger}P < 0.05$ versus mice treated with nondiabetic BM-MNCs; $^{\#}P < 0.05$, ## $P < 0.01$ versus PBS-injected diabetic animals. See legend to Figure 1.

mice. Low-density mononuclear cells were then isolated by density gradient centrifugation with Ficoll as previously described.⁴

EPC Differentiation Assay

Immediately after isolation, 5×10^6 bone marrow cells were plated on 35-mm cell culture dishes coated with rat plasma vitronectin (Sigma, St Quentin Fallavier, France) and gelatin (0.1%) and maintained in endothelial basal medium (EBM2, BioWhittaker, Paris, France). After 4 days in culture, nonadherent cells were removed and adherent cells underwent immunochemicals analysis as previously described.⁴

To detect the uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (AcLDL-Dil), cells were incubated in medium containing AcLDL-Dil (Tebu, Le Perray en Yvelines, France) at 37°C for 1 hour. Cells were then fixed with 2% paraformaldehyde and incubated with FITC-labeled BS-1 lectin (Sigma). Dual-stained cells positive for both AcLDL-Dil and BS-1 lectin were judged to be EPCs, and they were counted per well, as previously described.^{4,18} Antibody directed against CD18 (Sigma) was also used to confirm that cultured EPCs did not derive from monocytes/macrophages. Five replicates were done for each experimental conditions. Three independent investigators evaluated the number of EPCs per well by counting three randomly selected high-power fields under fluores-

Figure 3. A: Ischemia-induced changes in hindlimb blood flow monitored *in vivo* by laser Doppler perfusion imaging performed at day 10 after ischemia in nondiabetic mice treated with BM-MNCs isolated from nondiabetic or diabetic mice. In color coded images, normal blood flow is depicted in red. A marked reduction in blood flow of ischemic hindlimb is depicted in blue. **B:** Quantitative evaluation of blood flow expressed as a ratio of blood flow in ischemic foot to that in nonischemic one. Values are means $\pm$ SEM, $n = 7$ per group. * $P < 0.05$ versus PBS-injected nondiabetic animals. † $P < 0.05$ versus mice treated with nondiabetic BM-MNCs, # $P < 0.05$ versus PBS-injected diabetic animals. See legend to Figure 1.

cence microscopy. Results are expressed as percentage of total number of cultured cells.

Angiogenesis Assay Using the Matrigel Model

Eight-week-old C57Bl/6 female mice, (Iffa-Credo) received in their back 0.5 ml subcutaneous injection of either Matrigel + 1×10^6 BM-MNCs isolated from nondiabetic mice, or Matrigel + 1×10^6 BM-MNCs isolated from diabetic mice. In a second set of experiments, BM-MNCs were labeled with a green fluorescent marker, PKH2-GL.¹³ After the injection, the Matrigel formed rapidly a subcutaneous plug that was left 14 days before removal.

On day 14, the mice were euthanized and the skins were pulled back to expose the Matrigel as previously described.¹⁹ Plugs were then removed and fixed with 3.7% formaldehyde at 4°C for 12 hours, embedded in paraffin, sectioned, and stained with Masson Trichrome. Matrigel plugs were sampled systematically, three successive sections of 5 μ m, at the top, middle, and bottom

of each plug, and then examined (10 $\times$, 20 $\times$, and 40 $\times$ magnification; Olympus BH-2, Leica).

Statistical Analysis

Results are expressed as mean $\pm$ SEM. One-way analysis of variance was used to compare each parameter. Post hoc Bonferonni's *t*-test comparisons were then performed to identify which group differences account for the significant overall analysis of variance. A value of $P < 0.05$ was considered as statistically significant.

Results

Physiological Data

Blood glucose level was 1.9-fold increased in diabetic mice compared to nondiabetic animals (13.1 ± 1.8 mmol/L versus 6.7 ± 1.1 mmol/L, $P < 0.05$). In addition, body weight was 1.1-fold reduced in diabetic mice when compared to nondiabetic animals (28.3 ± 0.7 g versus 31.2 ± 0.9 g $P < 0.05$). Treatment with BM-MNCs did not affect these parameters in either group (data not shown).

Effects of BM-MNCs Isolated from Diabetic Mice on Ischemia-Induced Neovascularization

Endogenous BM-MNCs have been shown to secrete angiogenic factors, to incorporate into foci of neovascularization, and thereby to increase new blood vessel formation in ischemic tissue.^{12,13} Impairment of ischemia-induced neovascularization in diabetes may be related to a decrease in pro-angiogenic potential of endogenous BM-MNCs. Hence, we analyzed the pro-angiogenic potential of diabetic and nondiabetic BM-MNCs in the nondiabetic ischemic leg.

Angiographic Score

Transplantation of BM-MNCs isolated from nondiabetic animals raised the ischemic/nonischemic angiographic score by 1.8-fold over that of nondiabetic animals receiving PBS ($P < 0.05$) (Figure 1). Administration of BM-MNCs isolated from diabetic mice increased by 1.4-fold vessel density when compared to PBS-injected nondiabetic animals ($P < 0.05$). However, angiographic score was still 1.4-fold lower in mice treated with BM-MNCs isolated from diabetic animals compared to those treated with BM-MNCs isolated from nondiabetic animals ($P < 0.05$).

Capillary Density

Capillary density analysis after fibronectin staining correlated with microangiographic data (Figure 2). Injection of BM-MNCs isolated from nondiabetic animals enhanced ischemic/nonischemic capillary density ratio by 2.7-fold over that of nondiabetic animals receiving PBS ($P < 0.01$). Administration of BM-MNCs isolated from diabetic mice increased by 1.7-fold capillary number when compared to PBS-injected nondiabetic animals

Figure 4. **A:** Representative images of EPCs isolated from bone marrow of nondiabetic and diabetic mice with femoral artery ligation. EPCs were characterized as adherent cells with double-positive staining for AcLDL-Dil and BS-1 lectin. **B:** Representative phase contrast image showing spindle-shaped attaching cells from bone marrow of nondiabetic ischemic mice. **C:** Representative images of EPCs isolated from bone marrow of ischemic nondiabetic mice showing that most of these cells were negative for CD18. **D:** Quantification of AcLDL-Dil and BS-1 lectin-positive cells in nondiabetic and diabetic mice. Values are means $\pm$ SEM, $n = 5$ per group. *** $P < 0.001$, versus nondiabetic mice with femoral artery ligation.

Table 1. Capillary Density Expressed as a Ratio of Capillary Number to Fiber Number

	Ischemic	Nonischemic	Ischemic/nonischemic
Nondiabetic mice			
PBS	1.61 ± 0.22	5.11 ± 0.60	0.31 ± 0.04
Nondiabetic BM-MNCs	4.32 ± 0.51**	5.32 ± 0.71	0.81 ± 0.10**
Diabetic BM-MNCs	2.64 ± 0.41*†	5.23 ± 0.61	0.5 ± 0.07*†
Diabetic mice			
PBS	0.92 ± 0.074	4.81 ± 0.71	0.19 ± 0.06
Nondiabetic BM-MNCs	2.94 ± 0.40##	4.92 ± 0.83	0.60 ± 0.09##
Diabetic BM-MNCs	1.94 ± 0.32*†	4.71 ± 0.82	0.42 ± 0.06*†

* $P < 0.05$, ** $P < 0.01$ versus PBS nondiabetic mice; † $P < 0.05$ versus nondiabetic BM-MNCs; # $P < 0.05$, ## $P < 0.01$ versus PBS diabetic mice.

($P < 0.05$). However, the capillary number ratio was still 1.6-fold lower from that observed in mice treated with BM-MNCs isolated from nondiabetic animals ($P < 0.05$).

Laser Doppler Perfusion Imaging

Microangiographic and capillary density measurements were associated with changes in ischemic foot blood flow (Figure 3). Administration of BM-MNCs isolated from nondiabetic animals increased ischemic/non-ischemic blood flow ratio by 2.2-fold in reference to nondiabetic animals receiving PBS ($P < 0.05$). Injection of BM-MNCs isolated from diabetic mice also increased by 1.4-fold hindlimb blood flow recovery when compared to PBS-injected nondiabetic animals ($P < 0.05$) but to a lesser extent from that observed in mice treated with BM-MNCs isolated from nondiabetic animals ($P < 0.05$).

Effects of Diabetic Vascular Environment on BM-MNC Pro-Angiogenic Potential

Vascular endothelial dysfunction induced by diabetes may modulate the ability of BM-derived cells to restore angiogenic function. Therefore, we analyzed pro-angiogenic potential of diabetic and nondiabetic BM-MNCs in diabetic ischemic leg.

Angiographic Score

Transplantation of BM-MNCs isolated from nondiabetic animals raised the angiographic score by twofold over that observed in diabetic animals receiving PBS ($P < 0.05$) (Figure 1B). Administration of BM-MNCs isolated from diabetic animals also increased by 1.4-fold vessel density when compared to PBS-injected diabetic mice ($P < 0.05$), but to a lower extent than that observed in diabetic mice receiving BM-MNCs isolated from nondiabetic animals (1.5-fold, $P < 0.05$).

Capillary Density

Transplantation of BM-MNCs isolated from nondiabetic animals raised the ischemic/nonischemic capillary number ratio by 2.4-fold over that of diabetic animals receiving PBS ($P < 0.01$) (Figure 2B). Diabetic mice receiving BM-MNCs isolated from diabetic animals also showed a 1.8-fold increase in capillary number ratio when compared to PBS-injected diabetic mice ($P < 0.05$). Nevertheless, diabetic BM-MNC-related effects was 1.5-fold lower from those observed with nondiabetic BM-MNC administration ($P < 0.05$).

Laser Doppler Perfusion Imaging

Transplantation of BM-MNCs isolated from nondiabetic animals raised the blood flow ratio by twofold compared to diabetic animals receiving PBS ($P < 0.05$) (Figure 3B). Diabetic mice receiving BM-MNCs isolated from diabetic animals also showed a 1.4-fold increase in blood flow ratio when compared to PBS-injected diabetic mice ($P < 0.05$). Nevertheless, diabetic BM-MNC-related effects was 1.4-fold lower from those observed with nondiabetic BM-MNC administration ($P < 0.05$).

Effects of Diabetes on BM Cell Differentiation into EPCs

BM-MNCs have been shown to differentiate into EPCs *in vitro*.^{4,20} Diabetes may reduce differentiation of BM-MNCs into EPCs leading to abrogated postnatal neovascularization.

BM cells from nondiabetic and diabetic mice were isolated, cultured and differentiated into EPCs. A subset of BM-derived cells plated on vitronectin attached and became spindle shaped. EPCs were then characterized as dual-stained cells positive for AcLDL-Dil and BS1-lectin. Only a few positive staining for the monocytic

Figure 5. A: Top, representative photomicrographs of Matrigel sections from Matrigel treated with BM-MNCs isolated from nondiabetic mice stained with Masson Trichrome at a magnification of 10×, 20×, or 40×. **Bottom**, representative photomicrographs of Matrigel sections from Matrigel treated with fluorescent-labeled BM-MNCs isolated from nondiabetic mice at a magnification of 10× or 20×. **B: Top**, representative photomicrographs of Matrigel sections from Matrigel treated with BM-MNCs isolated from diabetic mice stained with Masson Trichrome at a magnification of 10×, 20×, or 40×. **Bottom**, representative photomicrographs of Matrigel sections from Matrigel treated with fluorescent-labeled BM-MNCs isolated from diabetic mice at a magnification of 10× or 20×.

A) Non-diabetic BM-MNCs

B) Diabetic BM-MNCs

Figure 6. A: Ischemic/nonischemic angiographic score at 7 days following ischemic injury in diabetic and nondiabetic mice treated with or without PIGF. B: Ischemic/nonischemic capillary number at 7 days following ischemic injury in diabetic and nondiabetic mice treated with or without PIGF. C: Ischemic/nonischemic limb perfusion at 7 days following ischemic injury in diabetic and nondiabetic mice treated or not with PIGF. Values are means $\pm$ SEM, $n = 6$ per group. *** $P < 0.01$ versus untreated nondiabetic animals. ††† $P < 0.001$ versus untreated diabetic animals.

marker CD18 has been observed in our experimental conditions, suggesting that most of the Dil LDL/BS-1 lectin positive cells are not derived from monocytes/macrophages (Figure 4). The percentage of cells with Dil LDL/BS-1 lectin positive-staining was low in diabetic and nondiabetic animals without femoral artery ligation ($<5\%$, $n = 5$ for each group) (data not shown). Ischemia induced by right femoral artery ligation markedly increased the percentage of cells with double-positive staining for AcLDL-Dil and BS-1 lectin in nondiabetic animals (10-fold, $P < 0.001$ versus nonischemic nondiabetic animals). This effect was hampered by diabetes ($55 \pm 10\%$ versus $10 \pm 3\%$ in nondiabetic mice with femoral artery ligation versus diabetic mice with femoral artery ligation, $P < 0.001$) (Figure 4). In addition, nondiabetic and diabetic BM cells cultured in high glucose conditions did not display changes in their ability to differentiate into EPCs (data not shown).

Effects of Diabetes on BM-MNC-Induced Vascular-Like Structure

BM-MNCs may incorporate into blood vessels in ischemic leg and myocardium.^{12,13} Hence, diabetes may affect the ability of BM-MNCs to contribute to blood vessel formation and therefore may reduce the revascularization process.

In the Matrigel plug, administration of BM-MNCs isolated from nondiabetic mice formed numerous tube-like structures and the presence of erythrocytes was evidenced in the lumen, demonstrating the existence of a functional vascular structure (Figure 5). Injection of fluorescence-labeled nondiabetic BM-MNCs disclosed that fluorescence-positive cells were incorporated into tube-like structures. These results also suggest that new vessels formed in the Matrigel plug are composed from one part of injected labeled BM-derived cells and from the other part of endogenous cells which have migrated in the Matrigel. Conversely, administration of BM-MNCs isolated from diabetic mice induced formation of disorganized cell clusters. In addition, injection of fluorescence-labeled diabetic BM-MNCs showed that fluorescence-positive cells did not markedly contribute to tube-like structure.

Therapeutic Potential of PIGF Administration in Ischemic Diabetic Mice

Differentiation into EPCs

We first assessed the effect of PIGF treatment on the ability of BM-MNCs to differentiate into EPCs in the setting of ischemia. PIGF administration increased by 1.4-fold the percentage of cells with double-positive staining for AcLDL-Dil and BS-1 lectin in nondiabetic animals ($67.6 \pm 7.3\%$) in reference to untreated nondiabetic mice ($48.2 \pm 5.4\%$, $P < 0.05$). PIGF treatment strongly increased by sixfold the number of cells with double-positive staining in diabetic mice ($64.1 \pm 7.9\%$ versus $11.2 \pm 1.9\%$ in PIGF-treated and untreated diabetic mice, respectively, $P < 0.001$).

Neovascularization Reaction

We next determined the therapeutic potential of PIGF treatment on diabetes-induced impairment of the neovascularization reaction. In nondiabetic animals, infusion of PIGF increased by 1.5-, 1.4-, and 1.6-fold the angiographic score, capillary number, and limb blood flow, respectively, when compared to untreated nondiabetic animals ($P < 0.01$) (Figure 6). Interestingly, in diabetic mice, PIGF administration enhanced ischemic/nonischemic angiographic score, capillary number, and blood flow recovery by 1.9-, 1.5- and 1.6-fold, respectively, over that of untreated diabetic animals ($P < 0.001$).

Discussion

This study shows that type I diabetes reduced the ability of endogenous BM-MNCs to activate the neovascularization process in the ischemic hindlimb. This latter effect might result from the impairment of diabetic BM-MNCs to differentiate into EPCs and to participate in vascular-like structure formation. Finally, we demonstrated that PIGF infusion restored diabetic BM-MNC function *in vitro* and improved the neovascularization reaction *in vivo*.

In the present study, transplantation of BM-MNCs isolated from nondiabetic mice consistently augmented angiogenesis and collateral vessel formation in ischemic tissue of nondiabetic mice, as previously described.^{8,12,13} The putative mechanisms for the accelerated angiogenesis induced by transplanted BM-MNCs may include incorporation into vascular structures and local production of angiogenic ligands or cytokines.^{8,12,13} Interestingly, we demonstrated that administration of diabetic BM-MNCs increased the angiogenic process in the ischemic leg of nondiabetic mice to a lesser extent than that observed with nondiabetic BM-MNC injection. In an effort to elucidate the driving mechanisms that lie behind the decrease in diabetic BM-MNC pro-angiogenic functions, we analyzed the ability of BM-MNCs to differentiate into EPCs *in vitro*. We showed that diabetes decreased the number of bone marrow-derived differentiated EPCs. In addition, we also demonstrated that diabetic BM-MNCs were unable to participate in vascular-like structure formation in a subcutaneous Matrigel plug. Our results are in line with other findings showing that human EPCs from other types of diabetes (ie, type II) exhibited impaired proliferation, adhesion, and participation in the wound healing process.¹⁸ Taken together, these results underscore that the BM-MNC pro-angiogenic potential is affected in diabetes and that the altered function may participate in the abrogated neovascularization process observed in diabetes. BM-MNCs represent a heterogeneous population of cells; therefore, further studies are required to determine whether diabetic BM-MNC dysfunction is specifically related to dysfunction in diabetic BM-EPCs, other cellular populations, or both.

The functional capacity of the BM-derived cells may be governed by the endothelial cells and/or vascular environment in diabetes. However, the pro-angiogenic poten-

tial of nondiabetic and diabetic BM-MNC administration was similar in diabetic mice and in nondiabetic animals. Hence, vascular or cellular environment in diabetes does not affect BM-MNC-related effect, suggesting that diabetic environment is unlikely involved in the decrease in endogenous diabetic BM-MNC pro-angiogenic potential. Bone marrow-derived EPCs from young mice restore PDGF pathways and cardiac angiogenic function in the aging host, suggesting a therapeutic role for BM-derived cells administration in reversing the aging associated cardiovascular complications.²¹ We can also speculate that the role of nondiabetic BM-MNCs is likely not restricted to the restoration of vessel number in ischemic leg of diabetic mice and may have a therapeutic role in reversing the diabetes-induced changes in VEGF production or endothelial function.^{16,17,22} Endogenous diabetic BM-MNCs may also lack such capacity to reverse vascular/cell dysfunction induced by conditions related to the pathophysiology of diabetes.

Finally, we demonstrated that PIGF infusion increased the neovascularization reaction in ischemic hindlimbs of diabetic mice. Similarly, PIGF administration has been shown to increase postischemic neovascularization in nondiabetic mice.¹⁰ In addition, collateral artery growth is more significant in response to treatment with the VEGFR-1-specific ligand PIGF in comparison to the VEGFR-2-specific ligand VEGF-E, highlighting the potential for therapeutic strategy based on PIGF administration.²³ Several lines of evidence also suggest that PIGF enhances mobilization and recruitment of BM-derived cells. Hematopoietic stem cells appear to express VEGF-R1 and to be responsive to PIGF.²⁴ In addition, anti-VEGF-R1 suppresses recruitment of myeloid progenitors and transplantation of wild-type bone marrow rescues the impaired collateral growth in PIGF-deficient mice.²⁵ We have extended these previous studies, since we demonstrated that the ability of BM-MNCs to differentiate into EPCs was increased by PIGF administration. Interestingly, PIGF totally restored the diabetes-induced impairment in the ability of BM-MNCs to differentiate into EPCs. Such PIGF-related effects might subsequently participate in the enhanced neovascularization reaction observed in the ischemic leg of PIGF treated-diabetic mice. Taken together, these results offer a foundation for the development of strategies tailored to the treatment of BM-MNC dysfunction and underline the potential for therapeutic strategy based on PIGF administration in restoration of BM-MNC function and postischemic revascularization in the setting of diabetes.

In conclusion, the present study shows that endogenous BM-MNC differentiation into EPCs and pro-angiogenic activity were reduced in type I diabetes in the setting of ischemia. These alterations in BM-derived cell functions might participate in the abrogated neovascularization process observed in diabetes. We also demonstrate that PIGF constitute a potential candidate for therapeutic modulation of postischemic neovascularization in diabetes. Further studies are necessary to confirm these results in type II diabetes and to address the specific mechanisms driving altered BM-derived cell activity and PIGF-related effects.

References

- Carmeliet P: Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 2000, 6:389–395
- Arras M, Ito WD, Scholz D, Winkler B, Schaper J, Schaper W: Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. *J Clin Invest* 1997, 101:40–50
- Silvestre JS, Mallat Z, Tamarat R, Duriez M, Tedgui A, Levy BI: Regulation of Matrix Metalloproteinase activity in ischemic tissue by interleukin-10: role in ischemia-induced angiogenesis. *Circ Res* 2001, 89:259–264
- Mallat Z, Silvestre JS, Le Ricousse-Roussanne S, Lecomte-Raclet L, Corbaz A, Clergue M, Duriez M, Barateau V, Akira S, Tedgui A, Tobelem G, Chvatchko Y, Levy BI: Interleukin-18/interleukin-18 binding protein signaling modulates ischemia-induced neovascularization in mice hindlimb. *Circ Res* 2002, 91:441–448
- Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T: Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 1999, 5:434–438
- Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K, Shimada T, Oike Y, Imaizumi T: Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* 2001, 103:2776–2779
- Crosby JR, Kaminski WE, Schattelman G, Martin PJ, Raines EW, Seifert RA, Bowen-Pope DF: Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. *Circ Res* 2000, 87:728–730
- Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM: Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 1999, 85:221–228
- Kalka C, Masuda H, Takahashi T, Gordon R, Tepper O, Gravelleaux E, Pieczek A, Iwaguro H, Hayashi SI, Isner JM, Asahara T: Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. *Circ Res* 2000, 86:1198–202
- Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compennolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P: Revascularization of ischemic tissues by PIGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med* 2002, 8:831–840
- Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhardt D, Wang J, Homma S, Edwards NM, Itescu S: Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* 2001, 7:430–436
- Schattelman GC, Hanlon HD, Jiao C, Dodds SG, Christy BA: Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. *J Clin Invest* 2000, 106:571–578
- Shintani S, Murohara T, Ikeda H, Ueno T, Sasaki K, Duan J, Imaizumi T: Augmentation of postnatal neovascularization with autologous bone marrow transplantation. *Circulation* 2001, 103:897–903
- Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, Amano K, Kishimoto Y, Yoshimoto K, Akashi H, Shimada K, Iwasaka T, Imaizumi T: Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet* 2002, 360:427–435
- Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schumichen C, Nienaber CA, Freund M, Steinhoff G: Autologous bone-marrow stem-cell transplantation for myocardial regeneration. *Lancet* 2003, 361:45–46
- Rivard A, Silver M, Chen D, Kearney M, Magner M, Annex B, Peters K, Isner JM: Rescue of diabetes-related impairment of angiogenesis by intramuscular gene therapy with adeno-VEGF. *Am J Pathol* 1999, 154:355–363
- Waltenberger J, Lange J, Kranz A: Vascular endothelial growth factor-A-induced chemotaxis of monocytes is attenuated in patients with diabetes mellitus: a potential predictor for the individual capacity to develop collaterals. *Circulation* 2000, 102:185–190
- Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP, Gurtner GC: Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion and incorporation into vascular structures. *Circulation* 2002, 106:2781–2786
- Tamarat R, Silvestre JS, Duriez M, Levy BI: Angiotensin II angiogenic effect in vivo involves vascular endothelial growth factor- and inflammation-related pathway. *Lab Invest* 2002, 82:747–756
- Grant MB, May WS, Caballero S, Brown GA, Guthrie SM, Mames RN, Byrne BJ, Vaught T, Spoerri PE: Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. *Nat Med* 2002, 8:607–612
- Edelberg JM, Tang L, Hattori K, Lyden D, Rafii S: Young adult bone marrow-derived endothelial precursor cells restore aging-impaired cardiac angiogenic function. *Circ Res* 2002, 90:E89–93
- De Vriese AS, Verbeuren TJ, Van de Voorde J, Lameire NH, Vanhoute PM: Endothelial dysfunction in diabetes. *Br J Pharmacol* 2000, 130:963–974
- Pipp F, Heil M, Issbrucker K, Ziegelhoeffer T, Martin S, van den Heuvel J, Weich H, Fernandez B, Golomb G, Carmeliet P, Schaper W, Clauss M: VEGFR-1-selective VEGF homologue PIGF is arteriogenic: evidence for a monocyte-mediated mechanism. *Circ Res* 2003, 92:378–385
- Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin DJ, Zhu Z, Bohlen P, Witte L, Hendriks J, Hackett NR, Crystal RG, Moore MA, Werb Z, Lyden D, Rafii S: Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med* 2002, 8:841–849
- Carmeliet P, Moons L, Luttun A, Vincenti V, Compennolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vanden-driessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG: Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* 2001, 7:575–583